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Mice Deficient in IL-1β-Converting Enzym Are Defective in Production of Mature IL-1β and Resistant to Endotoxic Shock

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Summary

IL-1β-converting enzyme (ICE) cleaves pro-IL-1β to generate mature IL-1β. ICE is homologous to other proteins that have been implicated in apoptosis, including CED-3 and Nedd-2/Ich-1. We generated *ICE*-deficient mice and observed that they are overtly normal but have a major defect in the production of mature IL-1β after stimulation with lipopolysaccharide. IL-1α production is also impaired. *ICE*-deficient mice are resistant to endotoxic shock. Thymocytes and macrophages from the *ICE*-deficient animals undergo apoptosis normally. ICE therefore plays a dominant role in the generation of mature IL-1β, a previously unsuspected role in production of IL-1α, but has no autonomous function in apoptosis.

Introduction

Interleukin-1β (IL-1β)-converting enzyme (ICE) is the cysteine protease that generates the bioactive form of the proinflammatory cytokine IL-1β from its biologically inactive precursor (Black et al., 1988; Kostura et al., 1989). ICE is unrelated to previously studied proteases and is unusual because of its requirement for Asp in the P1 position of its substrates (Howard et al., 1991; Sleath et al., 1990). Recently, two genes with possible roles in apoptosis and embryogenesis were discovered that have products closely related to ICE: the Caenorhabditis elegans cell death gene *ced-3* (Yuan et al., 1993) and the mammalian gene *Nedd-2/Ich-1* (Kumar et al., 1994; Wang et al., 1994). ICE, CED-3, and Nedd-2/Ich-1 are members of a novel cytoplasmic cysteine protease family.

Human or rodent ICE is synthesized in cells of the monocytic lineage as an inactive 45 kDa precursor processed proteolytically to generate active enzyme comprising polypeptides of 20 kDa (p20) and 10 kDa (p10) (Miller et al., 1993; Thornberry et al., 1992). The 45 kDa precursor is the only form of the enzym readily detected in cells, suggesting that processing may b the rate-limiting regulatory step (Ayala et al., 1994). Cl avages of the ICE precursor occur at potential ICE-processing sites, and autoprocessing can occur in vitro (Ayala et al., 1994). It is not known, however, whether ICE or another enzyme of similar specificity is responsibl for the activational processing in cells.

Two groups recently reported the three-dimensional structure of human ICE complexed with peptidic inhibitors: ICE is a homodimer of catalytic domains, each of which contains a p20 and a p10 subunit. The catalytic residues in the active site are Cys-285 and His-237. Four other amino acids whose side chains form the P1 carboxylate-binding pocket (Arg-179, Gln-283, Ser-347, and Arg-341), as well the active site Cys and His, are conserved among the family members ICE, CED-3, and Nedd-2/Ich-1 (Walker et al., 1994; Wilson et al., 1994).

ICE has attracted interest as a target for novel anti-inflammatory drugs, because the cytokine that it activates, IL-1β, has pleiotropic proinflammatory effects (Dinarello, 1991). IL-1β is implicated in the pathophysiology of vari us diseases, including rheumatoid arthritis, septic shock, inflammatory bowel disease, and insulin-dependent diabetes mellitus (Dinarello and Wolff, 1993a). The concept of ICE inhibitors as useful drugs, however, is complicated by the fact that IL-1α, the product of a distinct but closely related gene, binds the same receptors as IL-1β and therefore shares its proinflammatory activities (Dinarello and Wolff, 1993a). ICE has no known role in regulating the production of IL-1α. Although IL-1β is generally produced at much higher levels than IL-1α in human inflammatory diseases (Dinarello and Wolff, 1993a), the potential valu of ICE antagonists, as compared with receptor antagonists that could block both forms of IL-1, remains questionable.

The role of ICE in the production of mature IL-1β has been demonstrated in cell culture and ex vivo, but is not yet well studied in animals. Cells not expressing ICE were unable to process pro-IL-1β unless cotransfected with ICE cDNA constructs (Cerretti et al., 1992; Young et al., 1988). A tetrapeptide aldehyde ICE inhibitor decreased the release of mature IL-1β from stimulated cells in whole human blood (Thornberry et al., 1992). Release of small amounts of the precursor form, pro-IL-1β, was not blocked by the same ICE inhibitor and instead may have been stimulated. This raises a concern that in vivo other proteases, including elastase and cathepsin G (Black et al., 1988; Hazuda et al., 1988), could generate bioactive IL-1β by extracellular cleavage of released pro-IL-1β. Such activation might b particularly likely at sites of inflammation where inflammatory processes cause secretion of various proteases.

ICE and other members of the ICE-related protease family have recently been implicated in apoptosis, or programmed cell death. Overexpression of ICE in a rat fibroblast cell line caused apoptosis, and this could be blocked eith r by the product of *bcl-2*, a mammalian oncogene that can prevent apoptosis, or by crmA, the cowpox virus pr t in that is a specific inhibitor of ICE (Miura et al., 1993). Th involvement of ICE or a related prot ase in n ur nal cell apoptosis was suggested by the observation that microinjection of crmA expression vectors into chicken dorsal root ganglion cells blocked apoptosis induced by nerve growth factor deprivati n (Gagliardini et al., 1994).

The rol of the ICE protease family in apoptosis is of great scientific interest, but raises additi nal doubt about

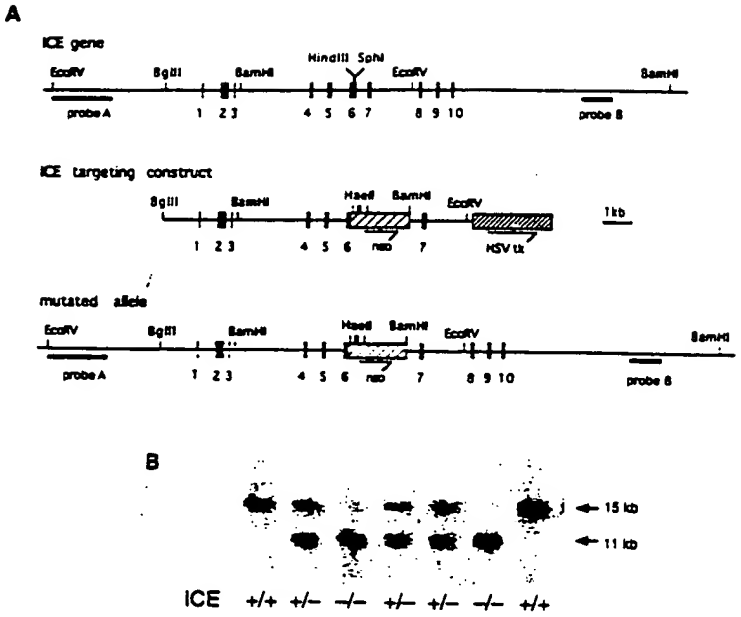

Figure 1. Disruption of *ICE* Gene by Homologous Recombination

(A) Structure of the *ICE* gene, the *ICE*-targeting vector, and the mutated locus following homologous recombination. Exons are depicted as closed boxes and are numbered. Relevant restriction enzyme sites are shown. Insertion of the *neo* resistance cassette between the HindIII and SphI sites in exon 6 leaves 13 nucleotides downstream. Diagnostic probes used for Southern blot analysis are shown.

(B) Southern blot analysis of tail DNA from wild-type, heterozygous, and homozygous *ICE*-deficient animals. DNA was digested with BamHI, and blots were hybridized to probe B. The wild-type allele is a 15 kb fragment, and the mutant allele is an 11 kb fragment.

the wisdom of selecting ICE as the target for novel anti-inflammatory drugs. The genetic approach pioneered by Capecchi (1989) makes it possible to address this concern, and the others mentioned previously, prior to the availability of inhibitors useful for pharmacologic studies in animals. Gene targeting in embryonic stem (ES) cells by homologous recombination can be used to generate mice with a null mutation in the *ICE* gene. Such animals would be valuable in the study of the role of ICE and IL-1 in health and disease. We report here results with *ICE*-deficient mice.

Results

Mice with a Null Mutation in the *ICE* Gene

A replacement-type targeting vector was constructed as outlined in Figure 1. The murine *ICE* gene is a single-copy gene with 10 exons (Casano et al., 1994). Exon 6 encodes Cys-284 and Gln-282, amino acids involved in catalysis and P1 substrate specificity, respectively. We cloned a genomic DNA fragment containing most of the *ICE* gene locus from the 129/Sv mouse strain and introduced between the HindIII and SphI sites of exon 6 the neomycin phosphotransferase (*neo*) gene under the control of the phosphoglycerokinase promoter. This insertion deleted 31 bp of ICE-coding sequence from the region of the active site. The change deleted amino acid Gln-282 and rendered out-of-frame the sequence beyond this residue. Introduction of a negative selection marker, the herpes simplex virus thymidine kinase (*tk*) gene, at the 3′ end of the construct enabled us t use positive and negative s lection (Mansour et al., 1988).

D3 ES cells (Doetschman t al., 1985) w re transfected with the targeting vector and selected with neomycin and gancyclovir. Cl nes were picked, and DNA was isolated from a portion of the cells and screened by Southern blot analysis. Three transfecti n experiments yielded 600 clones, of which only one clone had undergone homologous recombination. Cells from this clone were injected into blastocysts from C57BL/6 mice, and the injected blastocysts were implanted into pseudopregnant B6D2F1 mice. Ten male chimeras and one female chimera exhibiting more than 70% chimeric agouti patches were born. All eleven chimeras were able to transmit the mutation through the germline, as determined by polymerase chain reaction (PCR). As expected, 50% (86 of 173) of the offspring with agouti coat color derived from mating chimeras with C57BL/6 mice were heterozygous for the null mutation in the *ICE* gene. Heterozygous animals were mated, and approximately 24% (89 of 369) of the animals were homozygous for the null allele as determined by Southern blot analysis of tail DNA (Figure 1B).

ICE-Deficient Mice Are Overtly Normal

The *ICE*-deficient mice developed normally, appeared healthy, and were fertile. The average litter size (6.4) of *ICE*-deficient mice bred to date has been normal, with equal representation of both sexes. At the time of preparation of this manuscript, the oldest mice were 25 weeks old, and we had not observed any tumors.

Histopathological evaluation of all major organs including spleen, lung, heart, kidney, liver, adrenal gland, brain, gastrointestinal tract, pancreas, salivary gland, thymus, and testis from 8-week-old mice showed no abnormalities. *ICE*-deficient animals had normal numbers of leukocytes, erythrocytes, and platelets in the peripheral blood (data not shown). Percentages of various T cell subsets and B cells in freshly isolated thymuses, spleens, and lymph nodes w re examined by flow cytometry. Th re wer n significant differences in th $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$, and B cell populations in any of thes organs as compared with those of wild-type animals (data not shown).

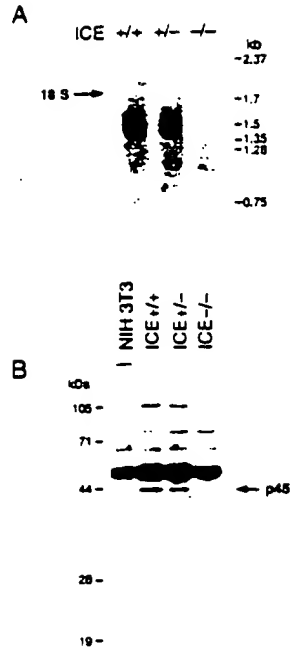

Figure 2. RNA and Protein Expression in *ICE*-Deficient and Control Mice

(A) Northern blot analysis of *ICE* RNA expression. RNA was isolated from spleens of wild-type (ICE+/+), heterozygous (ICE+/−), and homozygous (ICE−/−) *ICE*-deficient animals. Poly(A)$^+$ RNA was purified from 100 μg of total spleen RNA, then hybridized to a 1.2 kb XhoI–BamHI *ICE* cDNA fragment that recognizes the 1.6 kb *ICE* mRNA.

(B) Western blot analysis of ICE protein expression. The major form of ICE protein detected is the 45 kDa precursor protein (p45). In addition, expression of a polypeptide of 105 kDa, detected in the peritoneal macrophages, is reduced in *ICE* (−/−) lysates compared with *ICE* (+/+) and *ICE* (+/−) lysates. The 105 kDa protein was not detected in COS cell transfectants expressing human or murine ICE proteins (data not shown). We believe the 105 kDa protein may cross-react with the BBC2 antibody, or possibly its expression may be regulated by *ICE*.

Molecular Confirmation of the Null Allele in *ICE*-Deficient Mice

We were unable to detect the 1.6 kb *ICE* mRNA in the spleen of *ICE*-deficient mice by Northern blot analysis (Figure 2A), but we did observe a faint band of about 1.0–1.2 kb in three separate experiments that was also present in wild-type and heterozygous mice. Results from extensive PCR analysis using exon-specific primers suggest that this mRNA may result from cross-hybridization of the *ICE* probe to another member of the ICE superfamily (data not shown). As expected, Northern blot analysis using a *neo*-specific probe detected a low level of an mRNA (0.8 kb) in *ICE*-deficient and heterozygous mice, but not in wild-type littermates (data not shown). In further experiments, we extracted RNA from peritoneal exudate cells (PECs) and various organs, including brain, lung, heart, liver, adrenal gland, kidney, testis, and thymus, for analysis by reverse transcription (RT)–PCR and confirmed the absence of full-length *ICE* transcripts in all of these tissues (see Experimental Procedures).

To investigate ICE protein expression, we raised a polyclonal antibody against recombinant human ICE p32 protein (amino acids 120–404; Thornberry et al., 1992) and used it to probe Western blots of macrophage cell lysates. Macrophages were selected because this cell type preferentially expresses ICE protein, the predominant form being the 45 kDa ICE precursor (p45; Ayala et al., 1994; H. A., unpublished data). We demonstrated in preliminary experiments that the antibody detects mouse ICE p45 and p32, but noted that it also cross-reacts with a prominent mouse protein of 50 kDa. As shown in Figure 2B, p45 ICE precursor was readily detectable in macrophages from both wild-type and heterozygous littermates, but not in those from *ICE*-deficient mice. As an additional control, we showed that NIH 3T3 fibroblasts express the 50 kDa mouse protein but no p45 ICE precursor, as expected from previous work on the tissue specificity of ICE expression (Molineaux et al., 1993).

Apoptosis Occurs in *ICE*-Deficient Mice

As the *ICE*-deficient mice have no apparent gross abnormalities, we surmised that there were no significant defects in normal physiological processes involving apoptosis. For example, mammary gland involution occurs as a result of mammary epithelial cells undergoing apoptosis after the lactation period (Strange et al., 1992), and we observed that the postlactation involution of mammary glands was normal in the *ICE*-deficient females postpartum on gross examination.

We studied apoptosis induced by in vitro treatment in two cell types, macrophages and thymocytes. ATP treatment induces apoptosis as well as IL-1 release in mouse macrophages (Hogquist et al., 1991; Perregaux and Gabel, 1994). We show in Figure 3A that ATP induced DNA fragmentation in C57BL/6 and *ICE*-deficient macrophages. Thus, the *ICE*-deficient mice are not critically impaired in ATP-induced macrophage apoptosis.

Thymocytes undergo apoptosis in vitro after aging or exposure to agents such as dexamethasone and γ-irradiation. We measured apoptosis by determining the percent of hypodiploid cells after propidium iodide staining. As shown in Figure 3B, the percent of apoptotic cells was similar in the *ICE*-deficient and wild-type littermate mice with each of the three methods of induction studied. Similar results were obtained by DNA fragmentation analysis (data not shown). We also observed that the percent of CD4$^+$CD8$^+$ thymocytes, which are known to be susceptible to apoptosis, was similar after exposure to the above agents (data not shown).

Macrophages from *ICE*-Deficient Mice Are Defective in IL-1β Processing and Release

We assessed the processing and release of IL-1β ex vivo using thioglycollate-elicited peritoneal macrophages. Cells were stimulated with lipopolysaccharide (LPS) to induce expression of pro-IL-1β and then treated with ATP. Mature IL-1β levels in the medium were measured by enzyme-linked immunosorbent assay (ELISA). It was reported previously that the ATP treatment is necessary to trigger efficient processing and release of mature IL-1β from mouse

Figure 3. Apoptosis Ex Vivo in Macrophages and Thymocytes from *ICE*-Deficient and Control Mice

(A) DNA fragmentation induced by ATP. Macrophages were stimulated with LPS (1 μg/ml) for 4 hr then treated with or without ATP (5 mM) for 30 min. After removal of ATP, the cells were cultured for a further 8 hr, and then DNA was isolated. DNA was extracted from cells by using NTE buffer (pH 8.0; 100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA) containing 1% SDS and proteinase K (0.2 mg/ml), as described by Trauth et al. (1989). DNA samples were electrophoresed on 2% agarose gels containing ethidium bromide (0.5 μg/ml); those from C57BL/6 macrophages are labeled BL6.

(B) Apoptosis in thymocytes. Thymocytes from *ICE*-deficient homozygous (ICE−/−) and wild-type (ICE+/+) mice were incubated in vitro at 37°C for 18 hr with medium alone, medium with 1 μM dexamethasone, or with medium alone after pretreatment with γ-irradiation (5 Gy). Control cells were incubated at 4°C to inhibit apoptosis. The cells were then treated with 70% ethanol, RNase, and propidium iodide as described in Experimental Procedures. The stained cells were analyzed on a flow cytometer (FACScan) using CellFit software for estimating the percentage of hypodiploid cells.

macrophages (Hogquist et al., 1991; Perregaux and Gabel, 1994). We confirmed this observation and extended it to show that a selective ICE inhibitor, the tetrapeptide aldehyde known as Ac–YVAD–aldehyde, blocked the release of IL-1β in this assay (Table 1). Leupeptin, the tripeptide aldehyde known as LLR–aldehyde, was used as a specificity control and did not inhibit IL-1β release. This implies that the ATP-induced processing and release of mature IL-1β from mouse macrophages occurs via a mechanism dependent on ICE or another enzyme sensitive to the same inhibitor. The ATP treatment also triggers the release of IL-1α (Hogquist et al., 1991), and we additionally show in Table 1 that the release and extracellular accumulation of IL-1α is not significantly inhibited by the ICE inhibitor Ac–YVAD–aldehyde or by leupeptin.

The stimulated macrophages from *ICE*-deficient mice released barely detectable amounts of mature IL-1β (<20 pg/ml; Table 1). This is in striking contrast with the levels of 2000–4000 pg/ml released from macrophages of wild-type and heterozygous mice upon stimulation with LPS and ATP. Because the *ICE*-deficient macrophages failed to release mature IL-1β but nevertheless underwent apoptosis after the ATP treatment (see Figure 3A), this experiment also shows that apoptosis and release of mature IL-1β are independent processes. Interestingly, the extracellular accumulation of IL-1α was also markedly reduced from the *ICE*-deficient macrophages, to a level of about 25% of that from wild-type and heterozygous cells. This reduction was surprising, given that the Ac–YVAD–aldehyde ICE inhibitor failed to inhibit the extracellular accumulation of IL-1α from control macrophages.

Immunoprecipitations of IL-1α and IL-1β proteins were done on cell lysates and supernatants from peritoneal macrophages pulsed with [^{35}S]methionine (Figure 4). Analysis of the cell lysates showed that induction by LPS of the 31 kDa IL-1α and 34 kDa IL-1β precursors was similar in C57BL/6 and *ICE*-deficient macrophages. We also obtained similar results with wild-type and heterozygous macrophages (data not shown). Immunoprecipitations from the supernatants confirmed the ELISA results reported for IL-1α and IL-1β in Table 1. No 17 kDa mature IL-1β was detected in the supernatant of *ICE*-deficient macrophages after the 30 min treatment with ATP. A trace level of 17 kDa mature IL-1β was found in the supernatant of *ICE*-deficient cells upon further culture for 3 hr after removal of the ATP. Immunoprecipitations of IL-1α showed

Table 1. IL-1α and β Release from *ICE* (+/+), *ICE* (+/−), and *ICE* (−/−) Macrophages

Stimulation	IL-1α (pg/ml)			IL-1β (pg/ml)		
	ICE (+/+)	*ICE* (+/−)	*ICE* (−/−)	*ICE* (+/+)	*ICE* (+/−)	*ICE* (−/−)
LPS	5845 ± 860	3511 ± 313	1199 ± 118	4428 ± 36	1879 ± 184	<20
LPS plus YVAD	4162 ± 399	3135 ± 243	941 ± 30	833 ± 40	332 ± 60	<20
LPS plus Leupeptin	4241 ± 79	2971 ± 388	938 ± 12	4013 ± 262	2796 ± 123	<20

Thioglycollate-elicited macrophages were stimulated in vitro with LPS (1 μg/ml) and ATP (5 mM) (see Experimental Procedures). Levels of IL-1α and IL-1β in the medium at 3 hr after ATP addition were measured by ELISA. Where indicated, Ac–YVAD–CHO (YVAD) or leupeptin were present in the medium at 50 μM. *ICE* (+/+), *ICE* (+/−), and *ICE* (−/−) macrophages stimulated without LPS released <15 pg/ml IL-1α and <10 pg/ml IL-1β. The data shown are averages ± variation for supernatants from duplicate wells of macrophages. Similar results were obtained in three separate experiments.

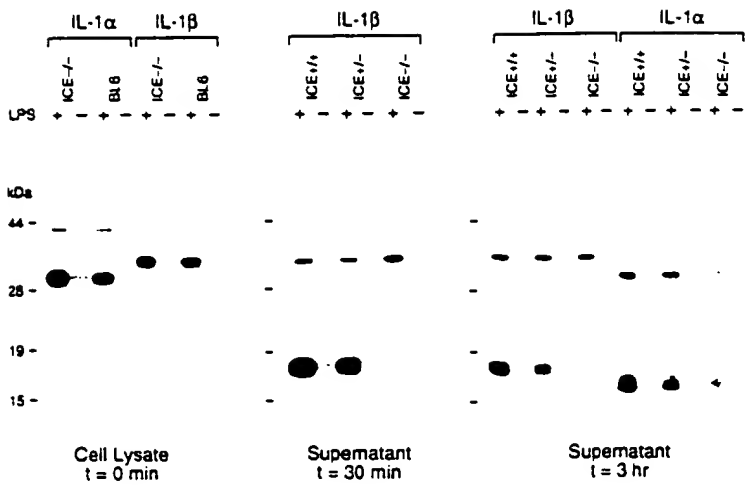

Figure 4. IL-1α and IL-1β Production by Macrophages from *ICE*-Deficient and Control Mice

Macrophages were stimulated with or without LPS (1 μg/ml) for 4 hr and pulse-labeled with [^{35}S]methionine during the last hour (see Experimental Procedures). Cell lysates were prepared before addition of ATP (time zero of the chase, t = 0 min). Supernatants were collected after the 30 min ATP treatment (t = 30 min) and after a further 3 hr culture following removal of ATP (t = 3 hr). Levels of IL-1α and IL-1β in the supernatants were quantitated by ELISA and were found to be similar to those shown in Table 1 (data not shown). In addition, cell lysates were prepared at t = 3 hr and immunoprecipitations of IL-1α and IL-1β performed (data not shown). This confirmed efficient induction of pro-IL-1α and pro-IL-1β in the cells from which the media supernatants were harvested. Lysates from C57BL/6 macrophages are labeled BL6.

that, at the 3 hr time point, the level of the 15 kDa mature form of IL-1α, as well as the level of the released 31 kDa precursor form, was significantly reduced in the supernatant of *ICE*-deficient cells compared with that in the supernatants of wild-type and heterozygote controls.

We conclude that macrophages from *ICE*-deficient mice have an extreme but not absolute defect in the production of mature IL-1β. In addition, there is an unanticipated major reduction in release and extracellular accumulation of IL-1α.

ICE-Deficient Mice Survive LPS-Induced Septic Shock and Produce No Detectable IL-1β or IL-1α In Vivo

Injection of high doses of LPS intraperitoneally into mice induces the massive systemic release of proinflammatory cytokines such as both types of IL-1 and TNFα. These cytokines are important in the pathogenesis of systemic inflammatory response syndrome (SIRS) and septic shock, which develops in the animals and results in their death (Dinarello et al., 1993b).

To investigate whether the *ICE*-deficient mice have a defect in production of cytokines in vivo, and to ask whether such a defect would lead to decreased SIRS, we administered LPS to mutant and control mice and then measured both survival and cytokine levels in plasma. We used a high dose of LPS (800 μg) that was found to cause 100% lethality in C57BL/6 mice in preparatory studies.

Preliminary experiments, using wild-type C57BL/6 and heterozygous *ICE*-deficient mice, showed high levels of IL-1β, IL-1α, TNFα, and IL-6 in plasma 4 hr after high dose LPS treatment (data not shown). Therefore, *ICE*-deficient mice (and wild-type littermates) were bled 4 hr after LPS injection. The plasma from groups of mice of each genotype and sex were pooled to obtain sufficient plasma for analysis. Representative data from one experiment are shown in Table 2. IL-1β was undetectable in the plasma of the *ICE*-deficient mice, although the cytokine was detectable at high levels in the control mice. Note that the results are listed separately by gender, because we found that male mice consistently had much lower concentrations of IL-1α and IL-1β after LPS injection than females. The concentrations of TNFα and IL-6 in the *ICE*-deficient mice were also moderately decreased compared with those of controls. Most surprisingly, we did not detect IL-1α in the plasma of *ICE*-deficient mice. IL-1α levels in wild-type female mice were approximately 250 pg/ml. Given the sensitivity of the ELISA that we used (30 pg/ml), we infer from these data that IL-1α levels in the *ICE*-deficient mice were more than 5-fold reduced compared with those of control animals after LPS injection.

Survival data are presented in Figure 5. All wild-type mice in three experiments succumbed to the high dose of LPS within 30 hr. Although we had noted a gender-specific difference in cytokine levels, there was no corresponding difference in time to death. By contrast, the *ICE*-deficient mice were highly resistant to the lethal effects of LPS, with all animals surviving beyond 30 hr and 70% of the mice surviving after 7 days. The first deaths of *ICE*-deficient mice occurred at around 45 hr in each experiment. *ICE*-deficient mice demonstrated signs of endotoxemia after LPS treatment, including lethargy, piloerection, and mild febrile shaking for the first few days after the LPS injection. These signs were milder than those observed in wild-type mice and perhaps can be attributed to the effects of TNFα in these animals.

Discussion

Gene targeting is a valuable method to evaluate the role of various genes in host defense and disease. Mice with disruptions in cytokine-related genes such as those for IL-2 (Schorle et al., 1991), IL-4 (Kühn et al., 1991), IL-6 (Kopf et al., 1994), IL-10 (Kühn et al., 1993), IFNγ (Dalton et al., 1993), IFNγ receptor (Huang et al., 1993), TNFβ (Togni et al., 1994), and p55 TNF receptor (Pfeffer et al., 1993; Roth et al., 1993) have been created. This report describes *ICE*-deficient animals; as yet we know of no published studies on the generation of mice deficient in IL-1α

Table 2. Plasma Cytokines in Endotoxic Shock

Cytokines (pg/ml)	Females		Males	
	ICE (+/+)	*ICE* (−/−)	*ICE* (+/+)	*ICE* (−/−)
IL-1β	493 ± 34	<20	126 ± 2	<20
IL-1α	253 ± 18	<30	62 ± 8	<30
TNFα	884 ± 56	627 ± 150	821 ± 12	562 ± 78
IL-6	520 ± 71	296 ± 16	209 ± 49	179 ± 12

ICE-deficient mice (*ICE* [−/−]) and wild-type mice (*ICE* [+/+]) were bled from the retro-orbital plexus 4 hr after LPS administration. The plasma from four mice of each gender and genotype was pooled for estimation of cytokines by ELISA. The data are means ± SD of two to four replicates from one pooled sample from each group.

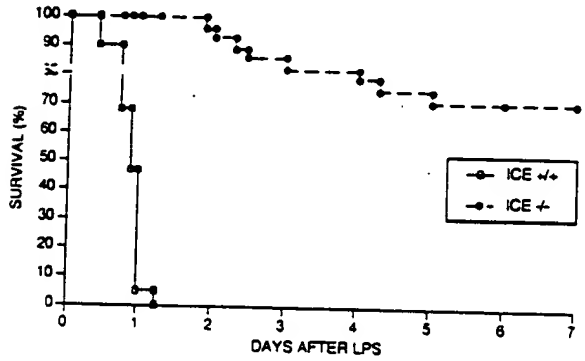

Figure 5. Survival of *ICE*-Deficient and Control Mice after Injection with High Doses of LPS

Survival of age-matched *ICE*-deficient homozygous (ICE−/−) and wild-type (ICE+/+) mice after injection with 800 μg of LPS intraperitoneally on day 0. The data shown are combined from three independent experiments, with a total of 28 *ICE* (−/−) and 19 *ICE* (+/+) mice. The survival of *ICE* (−/−) mice was significantly enhanced ($p < 0.001$ by χ^2 test) as compared with the *ICE* (+/+) mice.

or IL-1β. Therefore, the present work provides a significant example of mice with defects in IL-1.

The *ICE*-deficient mice are overtly normal. They have no apparent anatomical or developmental abnormalities as judged by gross examination of neonates and adults, or by histological analysis of all major organs. Hematopoietic and lymphoid cells appeared normal in number and distribution in the peripheral blood and lymphoid organs. We observed no evidence of any disease in the mice; spontaneous tumors have so far not occurred even in the oldest animals (25 weeks of age). The absence of any abnormality or pathology contrasts with results of null mutations in other cytokine genes, such as the lack of lymph nodes in TNFβ-deficient mice (Togni et al., 1994) and the occurrence of ulcerative colitis in IL-2- and IL-10-deficient mice (Kühn et al., 1993; Sadlack et al., 1993). One caveat, however, is that our animals are still young and have never been removed from specific pathogen–free (SPF) conditions. Their susceptibility to infectious agents that would be present in a less controlled normal environment is unknown. In IL-10-deficient mice, inflammatory bowel disease was less severe in animals maintained under SPF conditions.

Analysis of the *ICE*-deficient mice at the DNA, RNA, and protein levels confirmed that we had inactivated *ICE* gene function by targeted disruption. We did detect, at low level, an mRNA smaller than the major *ICE* transcript in splenic RNA from both the *ICE*-deficient and wild-type mice. This may be the product of an *ICE*-related gene, and we are now attempting to clone it for comparative analysis with *ICE* and other known family members.

While *ICE*-deficient mice have no gross abnormalities, they have a profound defect in their capacity to produce mature IL-1β. Peritoneal macrophages from the mutant mice, stimulated ex vivo by treatment with LPS and ATP, released only barely detectable amounts of mature IL-1β (less than 1% of control levels as measured by ELISA of cell supernatants). There was no decrease in pro-IL-1β synthesis or release as observed by immunoprecipitation. The small amount of mature IL-1β released by the macrophages from mutant mice was of the correct molecular weight, suggesting that specific cleavage of pro-IL-1β may occur in the mutant cells, although at a very low level. Macrophages from mice heterozygous for the *ICE* mutation produced about half the mature IL-1β level of those from wild-type mice, implying that the rate of IL-1β release from cells is proportional to *ICE* gene dosage. This suggests that ICE activity could be the rate-limiting step in regulating the amount of mature IL-1β released from stimulated macrophages.

We confirmed the defect in IL-1β production in vivo by observing that plasma from *ICE*-deficient mice injected with a high dose of LPS had undetectable levels of mature IL-1β. ICE is thus required for the dominant pathway of IL-1β maturation in response to LPS. The very low level of IL-1β production detected in cells ex vivo from the *ICE*-deficient mice must be generated by a different mechanism, possibly by extracellular proteolysis of pro-IL-1β released from cells. Alternatively, another member of the ICE protease family may inefficiently process pro-IL-1β, and experiments to test this possibility are in progress.

An additional question we planned to address with *ICE*-deficient mice was the relative importance of IL-1α and IL-1β in inflammation. Our presumption was that *ICE*-deficient animals would be blocked in IL-1β but not in IL-1α production, and therefore the animals would serve as models for the action of IL-1β-selective drugs. To our surprise, *ICE*-deficient mice also have a significant defect in IL-1α production: IL-1α production ex vivo by macrophages from these mice was only 20%–25% of wild-type levels after stimulation with LPS and ATP. IL-1α was not detected in the plasma of LPS-treated mutant mice, implying at least

a 5-fold decrease in accumulation in vivo. The defect in IL-1α production was unexpected, because earlier studies suggested that ICE does not process pro-IL-1α (Howard et al., 1991). Further, we show here that the ICE inhibitor Ac–YVAD–aldehyde does not appreciably prevent extracellular accumulation of IL-1α from stimulated cells. Processing of pro-IL-1α is thought to be accomplished by a calpain-like IL-1α-converting enzyme (Carruth et al., 1991; Kobayashi et al., 1990).

There are several possible explanations for the reduced IL-1α release in *ICE*-deficient mice. Our preliminary experiments suggest that mature IL-1β does not regulate the release of IL-1α from LPS-stimulated cells (data not shown). Therefore, we favor the idea that ICE is involved directly in the processing of IL-1α, its release, or both. Such roles could be independent of the catalytic activity of ICE and instead involve a cofactor function in IL-1α maturation. Further experiments are in progress to study this mechanism.

Another reason for developing *ICE*-deficient mice was to assess the potential role for this enzyme in apoptosis. Our results clearly prove that animals without *ICE* have no readily demonstrable defect in apoptosis. The absence of apparent abnormalities in the *ICE*-deficient mice argues strongly against a necessary function for ICE in apoptosis. If ICE played a required role in apoptosis during development, the *ICE*-deficient mice should have gross abnormalities in brain, gut, lymphoid, and neuronal tissues. At the cellular level in lymphoid tissues, there were no abnormalities in the numbers of B cells or T cell subsets, whereas abnormalities in B and T cell populations were reported in lymphoid tissue of mice transgenic for *bcl-2* (McDonnell et al., 1989; Sentman et al., 1991), a gene known to suppress apoptosis (Vaux et al., 1988). Macrophages and thymocytes from *ICE*-deficient mice retained their capacity to undergo apoptosis upon ex vivo stimulation by several different signals. This contrasts with mice that are p53 deficient or overexpress Bcl-2, both of which have defects in their apoptotic pathways that are discernible in vitro (Lowe et al., 1993; Sentman et al., 1991).

Transfection of cDNA vectors that encode ICE into a rat embryo fibroblast cell line induced apoptosis (Miura et al., 1993). This does not prove that ICE is a normal element in the apoptotic pathway, because unregulated ICE expression could be replacing a function normally served by a related enzyme with similar substrate specificity. Other members of the ICE family, namely the C. elegans gene *ced-3* (Yuan et al., 1993) and human *NEDD-2/ICH-1*, also caused apoptosis (Kumar et al., 1994; Wang et al., 1994) when transfected into rodent fibroblasts. While the natural substrates of CED-3 and NEDD-2/ICH-1 are not known, the conservation of all of the amino acids whose side chains determine the P1 substrate–binding pocket in the ICE X-ray structure (Walker et al., 1994; Wilson et al., 1994) suggests that all ICE family members are Asp-specific proteases. Granzyme B, a serine protease that is unrelated to ICE but shares the specificity for cleaving substrates after aspartyl residues, also induces apoptosis and plays a critical role in the cytotoxicity of natural killer cells and cytotoxic T cells (Hudig et al., 1993). These considerations suggest to us that there is a common substrate present in cells that, when cleaved by an appropriate Asp-specific protease, can cause apoptosis. ICE can accomplish such cleavage if overexpressed in fibroblasts, but the results with *ICE*-deficient mice argue that ICE is not involved in normal apoptotic pathways. We cannot, however, exclude the more complex theory that there are two or more proteases that function redundantly in the induction of apoptosis such that elimination of *ICE* alone has no effect. To test this possibility would require additional mice deficient in *ICE* homolog genes, and the mating of strains to produce mice with mutations in two or more family members.

Gagliardini et al. (1994) showed that transfection with crmA, a cowpox virus serpin known to inhibit ICE (Ray et al., 1992), could prevent apoptosis of chicken dorsal root ganglion neurons induced by nerve growth factor deprivation in vitro. This result does not conflict with the absence of an apoptosis defect in *ICE*-deficient mice if one proposes that crmA was acting by inhibiting an ICE homolog rather than ICE itself. The possibility that crmA may have inhibited an ICE homolog involved in neuronal apoptosis is further supported by the recent identification of a novel chicken protease, prICE, that resembles ICE but is implicated in apoptosis (Lazebnik et al., 1994).

The most striking result reported in this paper is that *ICE*-deficient mice are markedly resistant to the lethal effects of endotoxin. We used a high dose of LPS to induce SIRS. Whereas all the wild-type mice died within 30 hr after injection, all *ICE*-deficient mice studied so far have survived for at least 45 hr, and only 30% of animals died during the next 5 days. No IL-1β or IL-1α was detectable in the plasma of the mutant mice. The levels of TNFα and IL-6 were somewhat reduced compared with those in wild-type mice. The reasons for the delayed mortalities in *ICE*-deficient mice injected with LPS are unclear. The deaths may not relate directly to LPS-induced pathology. Rather, they may reflect other consequences of diminished IL-1 production, for instance, increased susceptibility to infection, either from endogenous gut flora or associated with the experimental protocol, which involved retro-orbital bleeding to obtain blood samples for cytokine measurements. The survival of *ICE*-deficient mice after a high dose of LPS is similar to the protection afforded by the IL-1 receptor antagonist (IL-1RA) (Alexander et al., 1991). The *ICE*-deficient mice are thus different from the IFNγ receptor–deficient and p55 TNF receptor–deficient mice, which are susceptible to high dose LPS-induced lethality (Car et al., 1994; Rothe et al., 1993).

Current hypotheses for the pathogenesis of septic shock are that microbial products such as LPS induce massive production and release of TNFα, which in turn induces IL-1 production and release by macrophages (Dinarello et al., 1993b). In human sepsis, the species of IL-1 detectable in plasma is exclusively IL-1β. Both TNFα and IL-1 have profound effects on vascular endothelial cells, leading to cell adhesion, vascular leakage, and shock. Neutralization of either TNFα or IL-1β prevents lethality in animal models of sepsis (Dinarello et al., 1993b). The present studies extend these observations and suggest that ICE inhibitors

could be useful in reducing mortality in human sepsis. Moreover, as SIRS is an extreme and acute example of systemic inflammation, ICE inhibitors may have th rapeutic potential in other inflammatory diseases, such as rheumatoid arthritis, in which IL-1β plays a significant role.

Experimental Procedures

Construction of the *ICE*-Targeting Vector

A partial murine *ICE* cDNA was isolated from a mouse macrophage cDNA library (Stratagene) by using a full-length human *ICE* coding sequence (provided by Dr. T. Ghayur). This cDNA was used to screen two mouse genomic DNA λ phage libraries made from the 129/Sv strain of mouse (Stratagene; J1 library provided by Dr. R. Jaenisch). The isolated murine *ICE* genomic clones were then subcloned into a plasmid vector, pBluescript (Stratagene), for restriction mapping, partial DNA sequencing, and construction of the targeting vector.

The *ICE*-targeting vector was constructed by using the plasmid pPNT (provided by Dr. R. Mulligan). A 2.2 kb SphI–NotI *ICE* fragment containing part of exon 6 and sequences downstream was isolated and subcloned into pBluescript at the BamHI and NotI sites by use of a SphI–BamHI adaptor made with two oligonucleotides with the following sequences: 5′-GATCCGAACCCCTTCGCATG-3′ and 5′-CGAAGGGGTTCG-3′. The 2.2 kb *ICE* fragment was then isolated as a BamHI–NotI fragment and the NotI end filled in with Klenow. This fragment was inserted into pPNT at the BamHI and EcoRI sites after filling in the EcoRI site, thus positioning it right after the *neo* gene on the 5′ end and right before the *tk* gene at the 3′ end. A 6.5 kb BglII–HindIII fragment containing *ICE* upstream sequences as well as exons 1–5 and ending in the middle of exon 6 was subcloned into pBluescript and subsequently excised out as a NotI–XhoI fragment and inserted 5′ to the *neo* gene in the pPNT31CE vector. In the final targeting construct, a 31 bp *ICE* sequence contained within the HindIII and the SphI sites was deleted from exon 6 (Figure 1A). This plasmid was linearized by NotI digestion.

ES Cell Culture and Transfection

D3 ES cells (supplied by R. Hynes) were cultured on a neomycin-resistant embryo fibroblast feeder layer grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml), nonessential amino acids, 100 μM β-mercaptoethanol, and 500 U/ml leukemia inhibitory factor. Medium was changed daily, and D3 cells were subcultured every 3 days. D3 cells (8×10^6) were transfected with 25 μg of linearized plasmid by electroporation (25 μF capacitance and 400 V) and cultured for the first 5 days in 2×10^{-6} M gancyclovir and 300 μg/ml neomycin and for the last 3 days in neomycin alone. Clones were expanded, some cells were frozen down, and DNA was isolated from the rest.

Southern Blot Analysis

ES cells or 0.5 cm of tail tissue were lysed in 100 mM Tris–HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 μg/ml of proteinase K. DNA was recovered by isopropanol precipitation and solubilized in 10 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA . After digestion with EcoRV and HaeII or BamHI alone, the DNA was resolved on a 0.8% agarose gel, blotted onto Hybond-N membrane, and hybridized at 65°C with probe A or probe B, respectively, described below. Blots were washed with 40 mM sodium phosphate buffer (pH 7.2), 1 mM EDTA, and 1% SDS at 65°C and exposed to X-ray film. Occurrence of homologous recombination could be confirmed readily, owing to the introduction of several HaeII sites and a BamHI site by the *neo* gene into the mutated exon 6 of the *ICE* gene. DNA was digested with EcoRV–HaeII and analyzed by use of probe A (Figure 1A), which binds a region proximal to the 5′ end of the targeting vector. The wild-type allele gave a fragment of approximately 13 kb and the mutant allele approximately 12 kb (data not shown). DNA was also digested with BamHI and analyzed by use of probe B (Figure 1B), which binds a region distal to the 3′ end of the vector. In this case, the wild-type allele gave a fragment of about 15 kb and the mutant allele a fragment of about 11 kb.

Generation of *ICE*-Deficient Mice

C57BL/6 female and male mice were mated, and blastocysts were isolated at 3.5 days of gestation. Cells from clone 164 were injected, 10–12 per blastocyst, and 7–8 blastocysts were implanted in the uterus of a pseudopregnant B6D2F1 female. Pups were delivered by cesarian section on day 18 and placed with a foster BALB/c mother. Male and female chimeras were mated with female and male C57BL/6 mice, respectively, and germline transmission was determined by the agouti coat color. After genotyping by Southern blot analysis, the heterozygous animals were mated with each other to generate homozygous mutant mice.

Northern Blot Analysis and RT-PCR Analysis of RNA

RNA was extracted from various organs of the mice by using 4 M guanidinium thiocyanate followed by centrifugation through 5.7 M CsCl (Sambrook et al., 1989). Poly(A)$^+$ RNA was isolated from 100 μg of total RNA and resolved on 1% formaldehyde agarose gels and blotted onto Biodyne nylon membrane (ICN Pharmaceuticals). Blots were hybridized with a ^{32}P-labeled 1.2 kb XhoI–BamHI *ICE* cDNA fragment. Alternatively, first-strand cDNA was synthesised through use of the GIBCO BRL Superscript system. PCR was performed with the Perkin Elmer Thermal Cycler and the following conditions: 95°C for 30 s (1 cycle); 94°C for 30 s, 54°C for 30 s, 72°C for 1 min (30 cycles); and 72°C for 5 min (1 cycle). PCR products were visualized by ethidium bromide on agarose gels.

The primers used and the results obtained are shown in Table 3.

Western Blot Analysis

Mice 8 weeks old were injected intraperitoneally with 1.5 ml of thioglycollate medium (Sigma). PECs were harvested 4–5 days later. Macrophages were purified from the PECs by adherence to plastic in RPMI 1640 without serum for 2 hr at 37°C. Macrophage cell lysates were separated on 10% SDS–polyacrylamide gels, then transferred to nitrocellulose filters (Amersham). Filters were probed with BBC2, a rabbit antibody to recombinant human ICE p32 protein (amino acids 120–404), at 1 μg/ml in PBS with 5% dried milk and 0.2% Tween 20. Detection was carried out by use of a secondary horseradish peroxidase–linked anti-rabbit antibody (Amersham) and the Amersham enhanced chemiluminescence system.

The recombinant human ICE p32 protein was expressed in Escherichia coli and purified, essentially as described for ICE p20 and p10 proteins by Walker et al. (1994). The rabbit polyclonal antibody raised to ICE p32, BBC2, was characterized by using COS cell transfectants expressing human ICE p32 and murine ICE p32 proteins, respectively.

Table 3. RT–PCR Analysis of *ICE* mRNA Expression

5′ Primer	3′ Primer	+/+	+/–	–/–
CCTGAGGGCAAAGAGGAAGC (*mICE* exon 2)	TCTGAAGGATTTTCTTTCCA (*mICE* exon 4)	+	+	+
	ATTTTCTTTCACTTTCACGG (*mICE* exon 5)	+	+	+
	AAGGAAAGTACTGTAAGAAG (exon 6, 5′ of *neo* insertion)	+	+	+
	CATGCCTGAATAATGATCACC (exon 6, 3′ of *neo* insertion)	+	+	–
	GAGCAGAAAGCAATAAAATC (*mICE* exon 7)	+	+	–
	AGCCTAAATTCTGGTTGTTC (*mICE* exon 9)	+	+	–
	GGCACGATTCTCAGCATAGG (*mICE* exon 10)	+	+	–

See text of this section for details.

These experiments showed that BBC2 recognized both human and murine ICE p32 proteins. In addition, the antibody cross-reacted with an endogenous 50 kDa COS cell protein in mock-transfected as well as ICE p32–transfected COS cells. Detection of the 50 kDa protein was not diminished by precleaning lysates with preimmune serum from the same rabbit used to generate BBC2.

IL-1 Release

Peritoneal macrophages were stimulated with LPS (E. coli strain O111: B4; Calbiochem) at 1 μg/ml in RPMI 1640 with 10% fetal calf serum for 4 hr at 37°C, then treated with ATP (5 mM, Sigma) for 30 min, as described by Hogquist et al. (1991). Fresh medium was added and the cells cultured for a further 3 hr. In some experiments, Ac–YVAD–aldehyde (Bachem Bioscience) or leupeptin (Sigma) was present in the medium at 50 μM. The levels of IL-1α and IL-1β in the medium were measured by ELISA kits from Genzyme Corporation and PerSeptive Diagnostics, respectively.

In preliminary experiments, we examined the kinetics of IL-1α and IL-1β release. There is a delay in release of IL-1α relative to IL-1β. Maximal release of IL-1α occurs between 2 and 3 hr after removal of ATP (data not shown). Cell viability was assesed by trypan blue exclusion and was ≥90% at the 3 hr time point.

Immunoprecipitation

Peritoneal macrophages were treated with or without LPS (1 μg/ml) for 4 hr and pulse-labeled with [^{35}S]methionine (200 μCi/ml, Du Pont) during hour 4. Labeled cells were washed with PBS and treated with ATP (5 mM) for 30 min, the medium was collected, and fresh medium was added and then harvested after a further 3 hr chase. Cell lysates were prepared either before addition of ATP (time zero of chase) or at 3 hr after ATP treatment (end of chase) by extraction with 1% Triton X-100, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl plus protease inhibitors (1 mM EGTA, 25 mM iodoacetamide, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride [PMSF], all from Sigma). Media samples were adjusted to 1% Triton X-100, 50 mM Tris–HCl (pH 8.0), plus protease inhibitors. In addition, a fraction of the media samples was saved to measure levels of IL-1α and IL-1β by ELISA (data not shown). Cell lysates and media samples were precleared with normal goat immunoglobulin (Sigma) and protein G–Sepharose (Sigma). Immunoprecipitations were performed with goat antibodies specific for mouse IL-1α or IL-1β (R and D Systems). Immunoprecipitates bound on protein G–Sepharose were washed five times with 1% Triton X-100, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 1 mM PMSF, then analyzed on 12% SDS–polyacrylamide gels. Similar results were obtained in two separate experiments.

Thymocyte Apoptosis

Thymocytes were incubated in RPMI with 10% fetal calf serum and supplements at a concentration of 2×10^6 cells/ml in 48-well tissue culture plates (Costar), at 37°C in a 5% CO_2 incubator. They were incubated with or without dexamethasone at 10^{-6} M or were γ-irradiated with 5 Gy prior to culture. Some cells were kept at 4°C to prevent apoptosis. The cells cultured in vitro were collected after 18 hr for analysis of apoptosis. Cells were fixed with 70% ethanol for 1 hr at 4°C, washed, and then treated with RNase (0.5 mg/ml) and propidium iodide (50 μg/ml) as described (Nicoletti et al., 1991). The cells were stored in the dark at 4°C until they were analyzed on the FACScan flow cytometer for propidium iodide fluorescence by use of CellFit software. The percent of cells with hypodiploid staining of the nuclei was taken as a measure of apoptosis.

LPS-Induced Septic Shock

Mice (8–10 weeks old) were injected with 800 μg of LPS from E. coli serotype O111:B4 (Calbiochem) intraperitoneally to induce high dose LPS-induced septic shock. The dose was based on published results and our own results obtained from studies on LPS-induced septic shock in C57BL/6 mice. The mice were monitored for signs of endotoxemia and lethality at least twice daily for 4 days and periodically thereafter. The animals were bled retro-orbitally (~100 μl) 4 hr after LPS injection, and plasma was pooled from animals of the same sex and genotype to obtain sufficient sample volume; it was then analyzed for cytokines. Cytokine estimations of murine IL-1β, IL-1α, and TNFα were done with ELISA kits from Genzyme Corporation. ELISA kits for murine IL-6 estimation were from Biosource International. Using the methods described in the kits and the dilutions used for the assays, we found the detection limits for IL-1β and IL-1α to be 20 pg/ml and 30 pg/ml, respectively.

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